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# *In vivo* imaging of trypanosomes for a better assessment of host–parasite relationships and drug efficacy

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ABSTRACT

The advances in microscopy combined to the invaluable progress carried by the utilization of molecular, immunological or immunochemical markers and the implementation of more powerful imaging technologies have yielded great improvements to the knowledge of the interaction between microorganisms and their hosts, notably a better understanding of the establishment of infectious processes. Still today, the intricacies of the dialog between parasites, cells and tissues remain limited. Some improvements have been attained with the stable integration and expression of the green fluorescence protein or firefly luciferase and other reporter genes, which have allowed to better approach the monitoring of gene expression and protein localization *in vivo*, *in situ* and in real time. Aiming at better exploring the well-established models of murine infections with the characterized strains of *Trypanosoma cruzi* and *Trypanosoma vivax*, we revisited in the present report the state of the art about the tools for the imaging of Trypanosomatids *in vitro* and *in vivo* and show the latest transgenic parasites that we have engineered in our laboratory using conventional transfection methods. The targeting of trypanosomes presented in this study is a promising tool for approaching the biology of parasite interactions with host cells, the progression of the diseases they trigger and the screening of new drugs *in vivo* or *in vitro*.



## 1. Introduction

Neglected diseases have gained particular attention from the economic powers these last years. These diseases are caused by parasites, bacteria or viruses considered until recently as those whose research support is very limited in comparison with the immense burden the diseases they cause, pose to humanity [1]. Today, one-sixth of the world's population suffers from at least one of these pathologies, as stated by the Global Health Observatory from the World Health Organization. They are not a "market" attractive to stimulate the large pharmaceutical companies in research and development (R&D) of new therapies. Not long ago, some efforts have revived the interest of pharmaceutical companies for "neglected" diseases, mainly targeting malaria, tuberculosis and AIDS (Acquired Immunodeficiency Syndrome). Since these three diseases are present in rich countries or affect travelers, these efforts are supported by financial incentives that include mixed partnerships between the public and private sectors. Unfortunately, this strategy has little impact on what is now described as the "most neglected diseases" such as those caused by parasites from the *Trypanosomatidae* family comprising Human and Animal trypanosomosis, leishmaniosis and Chagas Disease. The socio-economic impact of these diseases is very considerable, even if it tends to decrease in response to advances in the fight against insect vectors. The increased knowledge of cell biology and of the metabolic pathways of these trypanosomatids, as well as a more detailed knowledge of the interaction of these parasites with their hosts may carry new rational approaches to improve existing chemotherapies. In addition, the discovery of new chemotherapeutic targets against these microorganisms is a major goal of the Millennium that relies on the setting up of better and reliable medium and high throughput drug screening assays.

Some improvements to these goals have been anticipated in this last decade from the techniques of transfection developed for parasites using several reporter genes. For instance, they have highly contributed for the understanding of many crucial steps of the biology of the microorganisms and their relationship with their hosts at cellular and tissue levels. Pioneer studies on the expression of foreign intracellular reporter genes by trypanosomatids date back to the 90s. Early successful attempts started in 1991 with the transient expression of β-galactosidase (*β-gal*) and β-glucuronidase as reporter enzymes in *Leishmania (L.) tropica* [2]. The consideration of glycosome biogenesis to approach the energy source in trypanosomes was first studied in 1992 by the stable expression of firefly

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luciferase by procyclic forms of *Trypanosoma (T.) brucei* [3]. These studies, were followed by others using episomal or stable expression of other enzymes or green fluorescent protein (GFP) genes, to tackle gene expression and protein targeting in *Leishmania major* and *Trypanosoma cruzi* [4,5]. These reporter genes have been progressively used since then because they are sensitive, inexpensive, present low toxicity, and they are not expressed by mammalian cells. One of the best advantages of the utilization of transformed parasites with these genes is that they do not require any fixation and most of them allow an easy imaging *in vitro* and *in vivo*. For instance, different strains of *T. cruzi* were later transfected with an integrative vector that allows the expression of GFP or red fluorescent protein (RFP) showing the usefulness of these mutants to study several aspects of the infection such as the mechanisms of cell invasion, of genetic exchange and the differential tissue distribution in animal models [6]. These studies were followed by those using trypomastigote and amastigote forms of *T. cruzi* expressing the luciferase (*luc*) reporter gene illustrating the advantages of bioluminescent parasites for the imaging *in vitro* and *in vivo* [7]. The disease progression of experimental Human African Trypanosomosis and the systemic tropism, particular to the brain, were subsequently demonstrated by bioluminescent imaging using mouse adapted strains of *Trypanosoma brucei gambiense* [8]. Our recent studies with metacyclic trypomastigote forms of *Trypanosoma vivax* expressing luciferase have reinforced the presence of the parasite in the central nervous system of mice and consequently the brain commitment in the very late phases of the experimental infection [9]. Other colleagues, using intravital brain imaging and *Trypanosoma brucei brucei* or *Trypanosoma brucei rhodesiense* parasites, respectively expressing mOrange and tdTomato, have equally reported that human trypanosomes can invade the murine brain parenchyma during the early stages of infection before meningoencephalitis is fully established [10]. Interestingly, *T. b. brucei* expressing luciferase has allowed the determination of the commitment and dissemination of the parasites to the testis observation that may hamper the efficacy of treatments for sleeping sickness [11].

Other advances have been reported about the utilization of reporter genes in protozoan parasites for the discovery and development of new drugs and of the *in vivo* imaging for noninvasive visualization of biological processes (see [12] for a review). Some examples include the efficacy of drug screening based on the use of GFP-expressing *L. major* or *Leishmania donovani* [13,14], EGFP-, *β-gal*- or *luc*-expressing *Leishmania amazonensis* [15,16] and more recently on *T. cruzi* expressing the tomato protein [17]. In the present work we report some of the new tools we have developed in our laboratory to the well characterized strains of *T. cruzi* (CL and Y) and *T. vivax* (ILRAD 1392), aiming at better exploring their infectious process at cellular and tissue levels and to evaluate the effects of new therapeutic compounds *in vitro* and *in vivo*.

## 2. Material and methods

### 2.1. Mice and ethics

Seven to ten week-old male Swiss Outbred mice (CD-1, RJOrl: SWISS) (Janvier, France) were used in all experiments. All mice were housed in our animal care facility in compliance with European animal welfare regulations. Institut Pasteur is a member of Committee #1 of the Comité Régional d'Ethique pour l'Expérimentation Animale (CREEA), Ile de France. Animal housing conditions and the procedures used in the work described herein were approved by the "Direction des Transports et de la Protection du Public, Sous-Direction de la Protection Sanitaire et de l'Environnement, Police Sanitaire des Animaux" under number B 75-15-28, in accordance with the Ethics Charter of animal experimentation that includes appropriate procedures to minimize pain and animal suffering. PM is authorized to perform experiments on vertebrate animals (license #75-846 issued by the Paris Department of Veterinary Services, DDSV) and is responsible for all the experiments conducted personally or under her supervision as governed by the laws and regulations relating to the protection of animals. All animal work was conducted in accordance with relevant national and international guidelines.

### 2.2. Parasite strains and cell cultures

*T. (Dutonella) vivax* IL 1392 was originally derived from the Zaria Y486 Nigerian isolate [18]. These parasites have recently been characterized and are maintained in the laboratory by continuous passages in mice, as previously described in detail [19,20]. For *in vivo* experiments, mice were injected intra-peritoneally or sub-cutaneously with bloodstream forms of *T. vivax* ($10^2$ parasites/mouse). Parasitemia was determined, as previously described [19]. Cell culture-derived or axenic amastigote and trypomastigote forms from *T. cruzi* CL Brener (clone F11–F5) (www.dbbm.fiocruz.br/TcruziDB/clbrener.html) and Y strains were isolated from the supernatant of bulk cultures of green monkey Vero kidney cells previously infected with bloodstream trypomastigotes [21]. Epimastigote forms of *T. cruzi* are maintained by serial passages *in vitro*, as previously described [22]. Normal or infected Vero cell cultures were seeded in microplates or in flasks at $5 \times 10^4$ cells/ml in RPMI 1640 medium/5% FCS and kept at 37 °C, 5% $CO_2$.

*T. vivax* metacyclics were purified from epimastigote axenic culture according to [23] with minor modifications. Normal goat serum was added to culture supernatants to a final concentration of 10% and incubated for 30 min at 27 °C. During the incubation period, epimastigotes aggregated and formed clumps, while metacyclics remained swimming freely. The metacyclics were then separated from the epimastigote clumps by differential centrifugation using a swing out rotor (Jouan GR412, Fisher Bioblock Scientific, Strasbourg, France) for 5 min 200 g.

### 2.3. Vectors and transgenic parasites

The nourseothricin resistance gene (SAT) was amplified from pFX4.1SAT (Jena Bioscience) with the primers SAT-forward (5′ ATGGC GCGCCATGAAGATTTCGGTGATCC 3′) and SAT-reverse (5′ CGCCATGGTT AGGCGTCATCCTGTGCTCC 3′). The fragment obtained (539 bp) was digested with AscI and NcoI and inserted into AscI and NcoI sites of the p*Tv*LrDNA-luc vector [20] to replace the neomycin resistance gene to obtain p*Tv*LrDNA-luc-SAT (see Table 1). The gene encoding the near infrared fluorescent protein E2-Crimson [24], was amplified

**Table 1**
Plasmid characteristics.

| Plasmid | Species | Size (bp) | Promoter | 5′UTR trans-splicing region | Reporter gene | Intergenic region | Selectable marker | 3′ UTR |
|---|---|---|---|---|---|---|---|---|
| *pTvLrDNA-luc* [20] | *T. vivax* | 8390 | 18S r DNA[a] | *Tv*PRAC | Luciferase | *αβ-tub* | *Sat* | *βα-tub* |
| *pTvLrDNA-crim* | *T. vivax* | 7197 | 18S r DNA[a] | *Tv*PRAC | Crimson | *αβ-tub* | *Sat* | *βα-tub* |
| *pTcTREX-crim* | *T. cruzi* | 6929 | 18S r DNA[b] | *HX1-TcP2β* | Crimson | *5′-gapdh* | *Neo* | *3′-gapdh* |
| *pTcTREX-luc* | *T. cruzi* | 8164 | 18S r DNA[b] | *HX1-TcP2β* | Luciferase | *5′-gapdh* | *Neo* | *3′-gapdh* |
| *pTcTREX-gfp* [25] | *T. cruzi* | 7027 | 18S r DNA[b] | *HX1-TcP2β* | GFP | *5′-gapdh* | *Neo* | *3′-gapdh* |

[a] 1.8 kb fragment upstream the 18S rDNA of *T. vivax*.
[b] 0.8 kb fragment upstream the 18S rDNA of *T. cruzi*.

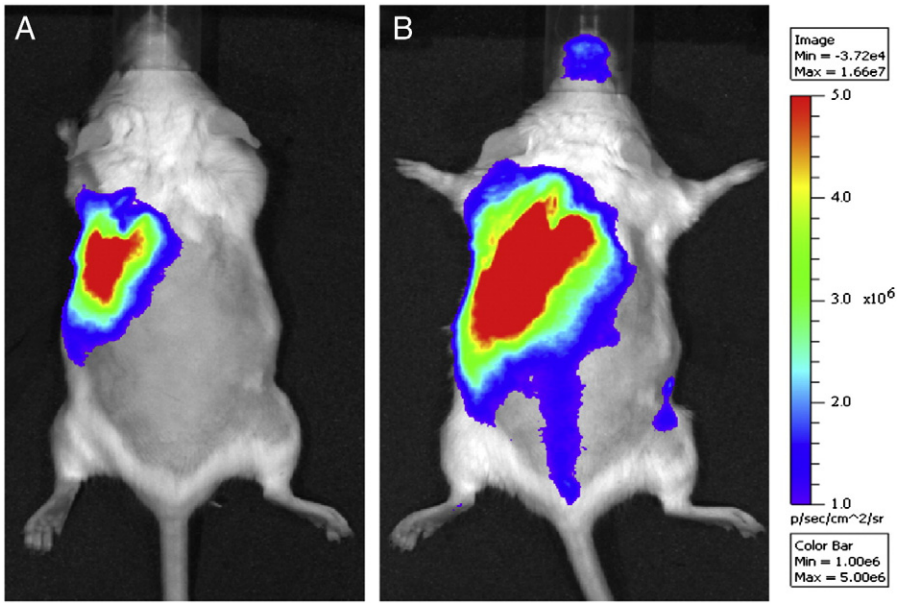



**Fig. 1.** Proliferation of *T. vivax* in the hypodermis after sub-cutaneous injection of culture-derived metacyclic parasites. Dorsal view of a representative Outbred mouse injected subcutaneously with $5 \times 10^4$ purified metacyclic forms of *Tv*LrDNA-luc parasites. Bioluminescence was measured at days 12 (A) and 13 (B). Color scale to the right of the pictures encodes for signal intensity (photons/s).

from pE2-Crimson (Clontech) with the primers Crim-forward 5′ GCG AAGCTTATGGATAGCACTGAGAACGTC 3′ and 5′ Crim-reverse GCGGG ATCCCTACTGGAACAGGTGGTGGCG 3′. The obtained fragment (683 bp) was digested with HindIII and BamHI and inserted into HindIII and BamHI sites of the p*Tv*LrDNA-luc-SAT to replace the luciferase gene to generate p*Tv*LrDNA-Crim-SAT or inserted into *T. cruzi* specific pTREX vector [25] digested with HindIII and BamHI to create p*Tc*TREX-Crim. Firefly luciferase reporter gene was purified from pGL3 basic vector (Promega) digested with HindIII and BamHI and inserted into vector pTREX previously digested with HindIII and BamHI to create pTREX-luc. All constructions were validated by sequencing or restriction cartography. The methods used to engineer *T. vivax* strain that stably expresses firefly luciferase (*Tv*LrDNA-luc) or E2-Crimson (*Tv*LrDNA-Crim) have been described elsewhere [20]. Briefly, nourseothricin was added to the cultures at a final concentration of 1 μg/ml to allow selection of recombinant *T. vivax*. Similarly, *T. cruzi* strain that stably expresses firefly luciferase (*TcTREX*-luc), GFP (*Tc*TREX-GFP) or E2-Crimson (*TcTREX*-Crim) was obtained by classical electroporation according to [26]. Total parasite genomic DNA was prepared from *in vitro* cultures with pure link genomic DNA (Invitrogen, Life Technologies, Villebon sur Yvette). Correct plasmid integrations were checked by PCR using standard techniques, oligonucleotide pairs flancking the integration regions and Dream Taq polymerase (Fermentas, Villebon sur Yvette, France).

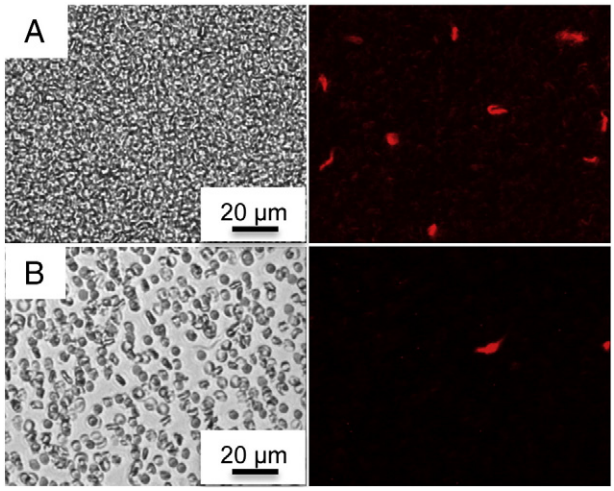



**Fig. 2.** Bloodstream forms of *T. vivax* transgenic parasites expressing E2-Crimson fluorescence. Undiluted (A) or diluted (1/10, B) blood samples from an infected mouse infected with *Tv*LrDNA-Crim were examined with a FLoid™ Cell Imaging Station (Molecular Probes Life Technology, France) using phase contrast (left panels) or red fluorescence channel (right panels).

### *2.4. Effects of specific trypanosomicidal drugs on parasite survival and multiplication*

Growth inhibition of *T. vivax* epimastigotes was monitored as follows. $5.10^5$ transgenic epimastigotes expressing the Luciferase or E2-Crimson were seeded per well, respectively on clear-bottomed or black- clear-bottomed 96-well microtiter plates (Corning, Life Sciences, France). Parasites rapidly adhere to the plastic surface and the supernatant was replaced by fresh growth medium containing different concentration of specific trypanosomicidal drugs such as homidium bromide (Ethidium), hygromycin, neomycin (G418), or benznidazole and paromomycin, a G418-analog. After 5 days media were discarded and adherent cells washed once in PBS in order to minimize background and tested for luciferase or fluorescence emission as described here below.

### *2.5. Luciferase and fluorescence assays in vitro*

A luciferase assay kit (Roche Molecular Biochemicals; Mannhein, Germany) was used to monitor luciferase expression. Serial dilutions of *T. cruzi* suspensions or *T. vivax* adherent cells were resuspended in 150 μl of cell lysis buffer. The lysates were then transferred into white, 96-well microplates (Dynex Technologies, Chantilly, France). Light emission was initiated by adding the luciferin-containing reagent, in accordance with manufacturer instructions. The plates were immediately transferred to the luminometer (Berthold $XS^3$ LB960; Thoiry, France) and light emission was measured for 0.1 s. Luminescence was expressed in Relative Light Units (RLU). E2-Crimson emitted fluorescence was

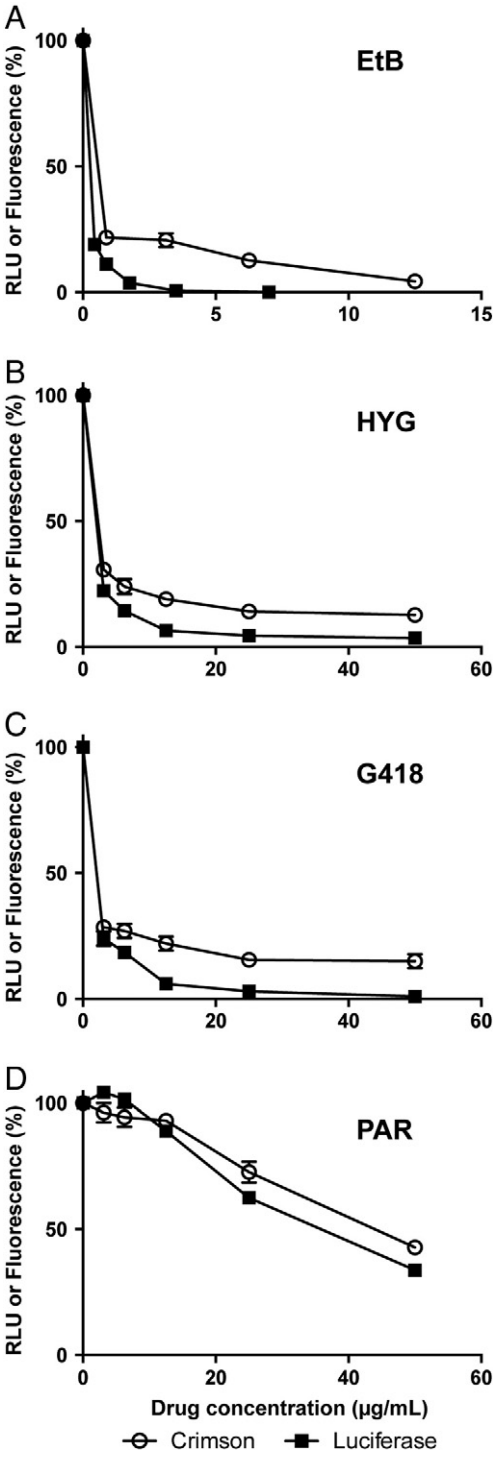



**Fig. 3.** *In vitro T. vivax* epimastigote inhibition of growth assay induced by trypanocidal drugs using fluorescent and luminescent parasites. $5 \times 10^5$ epimastigotes expressing E2-Crimson or Luciferase were inoculated per well into 96-well microplates and treated with Ethidium Bromide (Homidium bromide, EtB), Hygromycin (HYG), Geneticin (G418), or paromomycin (PAR) at different concentrations in TV3 medium. After 5 days, growth was determined by fluorescence (open circles) or luciferase (black squares) assay, as a relative percentage of the untreated strain. The results are the mean of duplicates and representative of two experiments.

measured in a SAFIRE II fluorometer (Tecan, Research Triangle Park, NC) using the black-clear-bottomed 96-well microtiter plates in the bottom-reading mode with excitation at 611 nm and emission at 646 nm.

### 2.6. In vivo bioluminescence imaging

Mice were inoculated intraperitoneally with luciferin (D-Luciferin potassium salt, Xenogen, California), the luciferase substrate, at a dose of 150 mg/kg before any bioluminescence measurements were made, as described [9]. They were anaesthetized in a 2.5% isoflurane atmosphere (Aerane, Baxter SA, Maurepas, France) for 5 min and kept in the imaging chamber for analysis. Emitted photons were acquired for 1 min by a charge couple device (CCD) camera (IVIS Imaging System Lumina, Caliper, Villepinte, France) set in high-resolution (medium binning) mode. The analysis was then performed after defining a region of interest (ROI). Total photons emitted from the image of each mouse were quantified using Living Image software (Xenogen Corporation, Almeda, California), and results were expressed as number of photons/s/ROI.

### 2.7. In vitro fluorescence imaging

Extracellular and intracellular fluorescent live parasite cultures were analyzed with a system for multicolor fluorescence FLoid™ Cell Imaging Station (Molecular Probes Life Technology, France) using phase contrast or fluorescence channels, or by confocal microscopy using a LMS700 (Carl Zeiss, France).

## 3. Results and discussion

### 3.1. Going beyond the development of tools to study T. vivax

#### 3.1.1. Multiplication of T. vivax in the hypodermis

With the use of bioluminescence imaging to track microorganisms *in vivo* it has become possible to quantify in real time the particularities, the abundance and the magnitude of an infectious process. For instance, as compared to other animal trypanosomes (*i.e. T. brucei* spp. and *Trypanosoma congolense*) *T. vivax* has the ability to be cyclically transmitted by *Glossina* spp. (tsetse) flies but also by other biting flies of the *Tabanidae* and *Muscidae* families that mechanically transmit the parasite among mammalian hosts. Regardless of the natural type of transmission (cyclical or mechanical), *T. vivax* is inoculated in the subcutaneous tissue and the infective forms join the bloodstream *via* the lymphatic system. We have recently constructed a *T. vivax* strain which stably expresses firefly luciferase (*Tv*LrDNA-luc) and that is fully virulent for immunocompetent mice [9]. The usefulness of real-time biophotonic analysis in the study and monitoring of the *T. vivax* infectious process *in vivo* was shown by tracking the infection dynamics and organ commitment after the inoculation of mice with *Tv*LrDNA-luc by the intraperitoneal route conventional used for experimental infections and the subcutaneous route that closely resembles the cyclic or mechanical infections conveyed by insects. Surprisingly, a longer prepatent period with a more delayed parasitemia onset was observed in mice injected subcutaneously with infective bloodstream forms of the *Tv*LrDNA-luc, as compared to that observed with mice inoculated by the intraperitoneal route. We show here that the same is observed when the inoculation is performed with axenic metacyclic forms of *Tv*LrDNA-luc that have differentiated *in vitro*. Therefore, the light emission of mice inoculated subcutaneously with metacyclic forms of *Tv*LrDNA-luc is confined to the skin near the inoculation site (Fig. 1). The increase in light emission in this region and the very circumscribed foci of photons shows that by this route of infection the parasite multiplication takes place close to the injection site before the parasites reach the bloodstream. Without the utilization of *Tv*LrDNA-luc and the noninvasive analysis of infection in living animals, the development of *T. vivax* in the skin would remain hidden from view or would depend on laborious or sophisticated microscopic exploration and requiring the sacrifice of several mice each day. Our previous studies have also pointed out that the infection causes a systemic disease and in the long term the parasite attacks the central

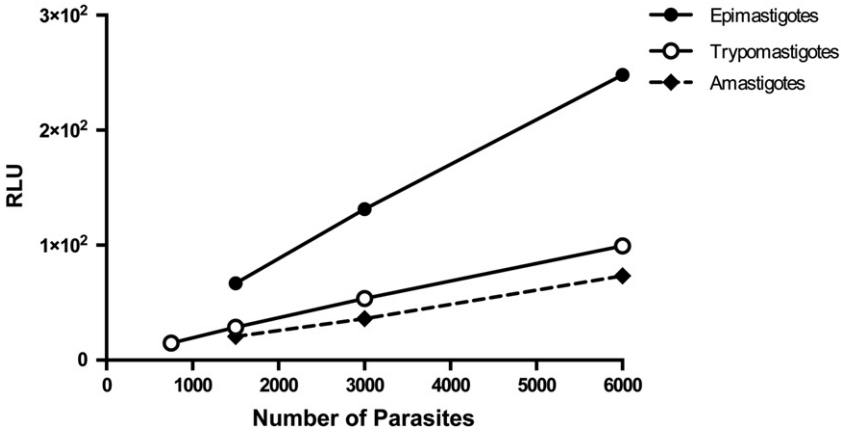



**Fig. 4.** Bioluminescence production by evolutive stages of *Tc*TREX-luc strain. Serial dilutions of *Tc*TREX-luc epimastigotes (black circles), trypomastigote forms (open circles) and amastigote forms (black diamonds) were measured *in vitro* for bioluminescent activity expressed in RLU (Relative Light Unit); the results are representative of two independent experiments.

nervous system of all animals that survived the parasitemia during the early phases and just a few days before death [9].

### 3.1.2. Brighter T. vivax: Validating far red fluorescence for in vitro imaging and drug screening

We then decided to explore the utility of *T. vivax* parasites stably expressing E2-Crimson protein, a protein that was evoked ideally for *in vivo* and stem cell applications due to its emission wavelength (646 nm) and its brightness [24,26]. The protein matures faster, and has high photostability, high solubility, and low cytotoxicity. Furthermore, the utilization of *in vitro* and *in vivo* imaging with fluorescent proteins is convenient over that of microorganisms expressing luciferase because they can be easily tracked in deep tissues and their detection does not require the intraperitoneal injection of any substrate to obtain a measurable signal. We have thus engineered a *T. vivax* strain that stably expresses the E2-Crimson (*Tv*LrDNA-Crim, see Table 1), as described in the Material and methods. The metacyclic infective forms of these recombinant parasites obtained *in vitro* maintain their infectivity to immunocompetent mouse strains and show the same parasitemia profiles over time that result in similar levels of mortality as wild type or *Tv*LrDNA-luc *T. vivax* (not shown). The fluorescence of the parasite was monitored at all life stages of *T. vivax* by microscopy. As can be seen in Fig. 2, bloodstream forms of *Tv*LrDNA-Crim show a bright red fluorescence distributed throughout all the cell body. Parasites have been maintained *in vitro* and *in vivo* for more than 3 months and stably expressing the fluorescence without antibiotic pressure.

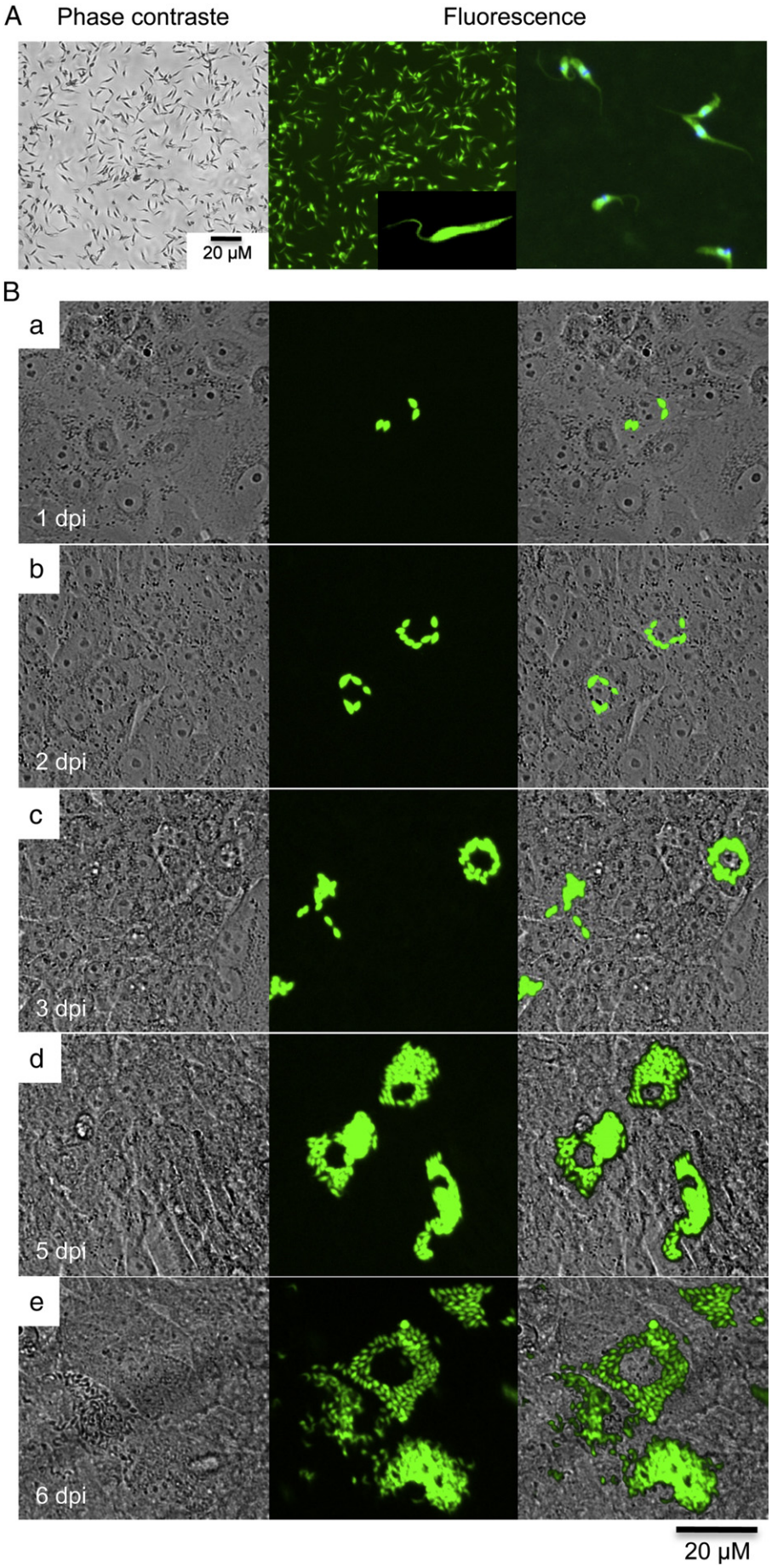
To verify the usefulness of *Tv*LrDNA-luc and *Tv*LrDNA-Crim in growth inhibition drug screening assays different concentrations of several cytotoxic compounds were added to parasite cultures in microplates and the luminescence and the fluorescence signal intensities were measured 5 days later as representative of *T. vivax* growth compared to nontreated wells. A dose dependent decrease of both luminescence and fluorescent signals has been evident as can be seen in Fig. 3A, B and C, respectively for Homidium bromide, Hygromycin and Neomycin. As can be observed, little variation is observed between the two experiments, figures are highly consistent and produce comparable results for *Tv*LrDNA-luc and *Tv*LrDNA-Crim. Noteworthy, the incubation of the parasites with the Paromomycin, an analog of G418 that is very toxic for *Leishmania* spp. is only partially effective in killing *T. vivax*, as ascertained by the weak relative decrease of the Crimson fluorescence and the luminescence signals (Fig. 3D). These data suggested that both transgenic parasites demonstrate the potential to be used in medium/high throughput assays of anti-*T. vivax* compounds *in vitro*. Experiments in progress aim at evaluating the utility of *Tv*LrDNA-Crim *in vivo* and will be a topic for a further study.

## 3.2. Building new outfits for T. cruzi

### 3.2.1. Gaining sensibility with luciferase and fluorescent reporter genes

Our laboratory has been involved for several decades with a systematic and global *in vivo* analysis of the infectious process caused by *T. cruzi*, mostly with immune activities triggered by the parasite for a better understanding of all mechanisms involved in infection aiming at novel therapeutical approaches [27–34]. However, the methods allowing for the precise detection of the parasite in the blood and in deep tissues, or else its interaction with host cells were until recently restricted to the optical microscopic appreciation of the parasite thus limiting for instance the evaluation of the efficacy of a treatment. To better study the mechanisms of host cell invasion, tissue distribution and the action of anti-parasitic compounds, our group and other groups have generated different strains of transgenic *T. cruzi* expressing luciferase or fluorescent reporter genes. For example, to improve research on the mechanisms involved in the mammalian pathogenesis or on the *T. cruzi* distribution inside the insect vector, Brazil and DM28C bioluminescent strains of *T. cruzi* were engineered, respectively using pBluescript (pBS-THT-x-T)- or pTREX- based plasmids [35,36]. On one hand, while these studies have shown a close correlation between total bioluminescence signal in the course of mouse infection and numbers of *T. cruzi* expressing the luciferase, they have precluded detailed assessment of the interaction of the parasite with the host at the cellular level [7]. On the other hand, the strategy revealed that it was not always possible to correlate the number of epimastigotes and the luminescence signal observed in the invertebrate *Rhodnius prolixus* alive and a more resolutive image of luminescent *T. cruzi* was only obtained in dissected insects [37]. However, the potentiality of the tool to study anti-parasitic compounds *in vitro* was clearly determined. In our laboratory, we have engineered CL-Brener *T. cruzi* strain that expresses luciferase, using the integrative hyperexpression pTREX

**Fig. 5.** Expression of green fluorescent protein by *Tc*TREX-GFP parasites. Epimastigote forms of *Tc*TREX-GFP were examined with a FLoid™ Cell Imaging Station (Molecular Probes Life Technology, France) using phase contrast (left panels) or green fluorescence (right panels); insert depicts closer slide views obtained with a confocal microscope; nucleus and kinetoplast of *Tc*TREX-GFP parasites were stained with DAPI (right panel)(A); trypomastigote forms of *Tc*TREX-GFP were used to infect Vero cells; cultures were examined daily for the growth of intracellular amastigotes throughout their release by the host cells. Days post infection (dpi) are depicted in the left inferior corner of the pictures, as follows 1 (a), 2 (b), 3 (c), 5 (d) and 6 (e) dpi. Images were obtained using a FLoid™ Cell Imaging Station (Molecular Probes Life Technology, France) using phase contrast (left panels) or green fluorescence channel (central panels); right panels represent merged pictures.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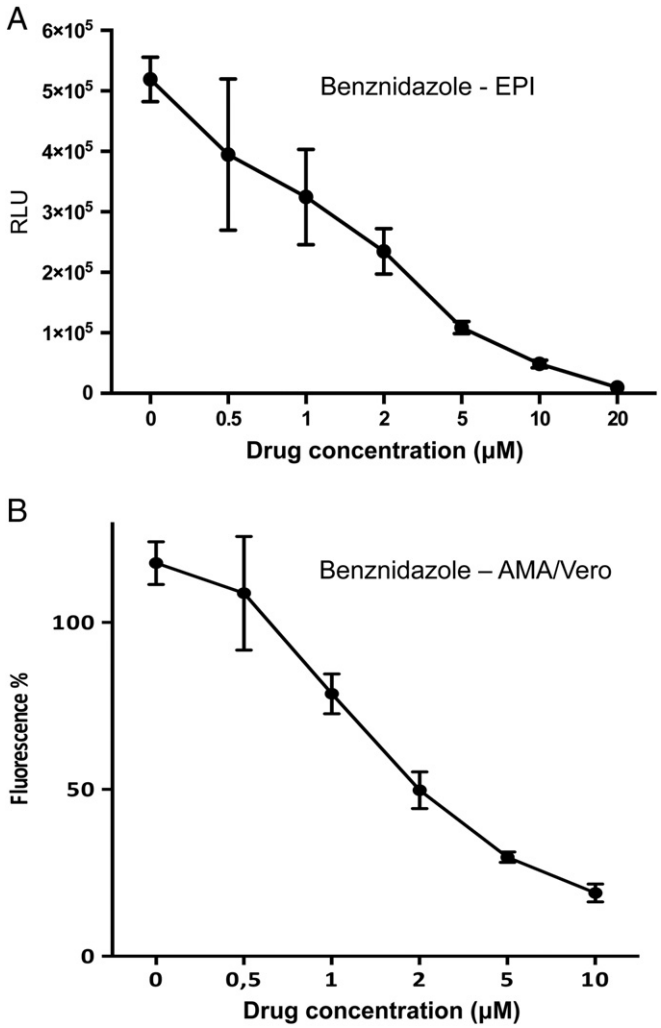


**Fig. 6.** Engineered *Tc*TREX-luc and *Tc*TREX-GFP are validated for drug screening strategies. Bioluminescence emission of *Tc*TREX-luc infected Vero cells, with increasing Benznidazole concentrations; the results are expressed in RLU and correspond to arithmetic means ±SD of the means (B). Fluorescence emission of *Tc*TREX-GFP infected Vero cells, with increasing Benznidazole concentrations; the results are expressed in % of fluorescence and correspond to arithmetic means ± SD of the means.

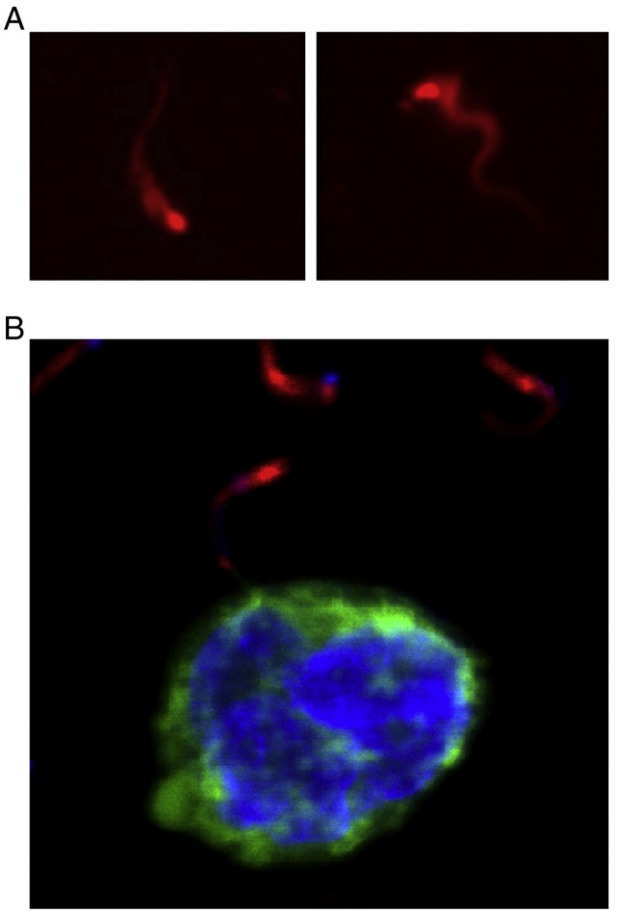


**Fig. 7.** Engineering *Tc*TREX-Crim strain for multicolor applications. Epimastigote (left panel) and trypomastigote (right) forms of Y strain of *T. cruzi* (A); *Tc*TREX-Crim parasites were pictured in the presence of human acute monocyte leukemia cell line (THP-1) stained with green fluorescence cell dye (pKH2) and DAPI, for contrast.

vector. The *Tc*TREX-luc parasites (see Table 1), showed strong emission of relative luciferase signal (RLU) in all life stages and in complete absence of antibiotic selection, as can be observed in Fig. 4.

To better explore the biochemical and photophysical properties of fluorescent proteins that can offer increased sensibility intracellularly as compared to luciferase, several trypanosomatids have been engineered to express GFP, RFP, EGFP, DsRed such as *T. brucei*, *Trypanosoma rangeli* and *Leishmania* spp., [15,38–40]. Similarly, we have expanded in the laboratory the CL-Brener strain of *T. cruzi* (clone F11F5) expressing GFP [25]. As can be observed in Fig. 5A the microscopic observation of the recombinant epimastigote forms of the *Tc*TREX-GFP reveals that the fluorescence emission by the transgenic parasites is highly homogeneous, as compared to the absence of fluorescence of WT parasites (not shown). Additional experiments using the infection of Vero cells with metacyclic *Tc*TREX-GFP parasites differentiated *in vitro*, show that the emission of fluorescence by infected cells rises throughout cellular infection, accompanying the increasing in numbers of intracellular parasites, as depicted in Fig. 5B, opening the way for evaluating the efficacy of new anti-parasitic drugs with a higher sensibility at the intracellular level.

*3.2.2. Exploring new imaging tools for drug screening against T. cruzi in vitro and in vivo*

In view of assessing if *Tc*TREX-luc and *Tc*TREX-GFP parasites could be used in the *in vitro* screening of trypanocydal properties of compounds anti-*T. cruzi*, we have set up new methods that rely on the analyses of light or fluorescent signals emitted by the parasites cultured in presence or in absence of Benznidazole (N-:benzyl-2-nitro-1-imidazol acetamide), a current medication against Chagas disease, using most accurate equipments for imaging in microplates, such as the Tecan and FLoid machines (see Material and methods). As anticipated, increasing concentrations of Benznidazole added to the culture of Vero cells infected with *Tc*TREX-luc trigger a progressive decrease of the photon emission (RLU), which correlates with the reduced cellular parasitism (Fig. 6A). Likewise, GFP signal proportionally decreases in Vero cell cultures infected with TcTREX-GFP parasites cultivated with increasing concentrations of the drug (Fig. 6B). These results validated these methodologies for future assays aiming at verifying the killing of intracellular amastigote forms of the parasite by new therapeutic leads against Chagas' disease previously identified in the laboratory [28,41].

An alternative vector, pROCK-Neo was used some years ago to facilitate the integration of fluorescent reporter genes into the tubulin locus of the *T. cruzi* genome resulting in stable transfections that can be also maintained without drug selection. This vector was previously used to introduce into several lineages of *T. cruzi*, the genes of GFP

(Colombiana, lineage I and JG, lineage II strains) or RFP (Tulahuen, lineage II and CL- Brener, hybrid strains) [6]. These combined uses of such parasites expressing two different markers in co-infection of Vero cells and the successful presence of *T. cruzi* expressing RFP and GFP constitute important technical tools to explore more in deep studies of genetic exchange in *T. cruzi*. More recently, CL-Brener transgenic parasites expressing β-Gal [42] or td-Tomato [17] using a pTREX backbone vector have been used to identify new drugs anti-*T. cruzi*. While the evaluation of drug efficacy using β-Gal — expressing *T. cruzi* requires the sacrifice of the experimental groups of mice for the analysis of parasitism rate in tissues mechanically disaggregated *ex-vivo*, screening assays using td-Tomato-expressing parasites made possible the analysis of parasite growth *in vitro* and *in vivo*. Thus, relatively low numbers of parasites expressing the td-Tomato protein are needed to inject the mice in the hind foot pads allowing the continuous quantification of the fluorescence signal over time. Aiming at improving deep-tissue imaging *in vivo* we have generated *Tc*TREX-Crimson parasites (*Tc*TREX-Crim, see Table 1), since both auto fluorescence and light scattering decrease substantially with this far-red shifted protein variant, as we observed for *T. vivax* (see here above). The first *T. cruzi* (Y) strain, transfected with *Tc*TREX-Crim vector is now available in the laboratory and has been submitted to antibiotic pressure and selection *in vitro*. Preliminary images of these parasites can be appreciated in Fig. 7. Likewise, *T. cruzi* of the CL strain stably expressing both luciferase and GFP reporter genes has been generated and selected *in vitro* and *in vivo*. These *Tc*TREX-Crim and *Tc*TREX-luc-GFP (*Tc*RoiGlum) parasites will be useful for whole-body imaging experiments and multicolor applications, for instance those implicating transgenic mouse lines expressing specific green fluorescent cell populations, such as macrophages and neutrophils [43–45]. Experiments are in progress in this sense.

## 4. Concluding remarks

The progresses of optical microscopy methodologies and *in vivo* imaging approaches involve the utilization of new reporter markers that facilitate multicolor studies of cellular interactions and tissue commitment by pathogens. One example is the rapid development of intracellular pathogens bearing new variants of fluorescent tags enabling a precise differentiation between the microorganism and other fluorescent markers used to transform host cells by animal transgenesis. These tools contribute to the advance of less laborious and highly performing techniques of imaging *in vivo* to evaluate infectious process progression or the efficacy of new chemotherapeutic compounds.

In more practical terms, arduous and inaccurate determination of parasitic burden in the blood of animals treated or untreated with chemical compounds by optical microscopy is close to be abandoned. In fact, the technique is presently replaced by the utilization of parasites expressing fluorescent proteins and can rapidly be counted using automated microplate assays and versatile image systems thus offering highly consistent sets of data, specifically for low parasite loads. On top of more comfortable analysis and the greater reproducibility they offer, the expression of luciferase or fluorescent reporter genes has allowed the setting up of medium/high throughput assays for the better evaluation of the toxicity of anti-parasitic drugs *in vivo*, tackling the effectiveness of a compound at deep tissue levels. Moreover, the efficient usage of parasites expressing fluorescent proteins supports the dynamic study of the same ongoing culture of parasitized cells for extended periods of time by using, for instance, an appropriate and fully integrated system for multicolor fluorescence cell imaging station like the one we used in this report. This type of apparatus contrasts with old-fashioned techniques based on the fastidious counting of stained cells on fixed slides. It is important to note that these recent strategies of imaging allow the detection of low numbers of parasites that would not be noticeable due to the limits of classic optical microscopy.

Finally, the novel intracellular applications of fluorescent proteins, their structure and corresponding functions, as well as the extensive range of spectroscopic features appropriate for high resolution techniques of imaging have been reviewed recently [46,47]. The versatility, photostability and, more importantly, the low cytotoxicity and the stable expression of far-red fluorescent reporter genes have solved some troublesome questions induced by the classical transgenesis of parasites. In fact, transfection of parasites may regularly give rise to a lack of virulence that has been explained either by the stress of the transfection programs used, by the long term maintenance of the mutants *in vitro*, or yet by the intrinsic toxicity of the fluorescent protein compromising the parasite viability *in vitro* and *in vivo*. For instance, the E2-Crimson, a fluorescent derivative of DsRed protein, does not present any toxicity for mammals and isolated cells and is one the most recent fluorescent proteins whose utilization is appropriate for imaging of living animals. Mainly this is due to its excitation and emission wavelengths above 600 nm, which have low absorption and light-scattering in tissues. This characteristic, associated to the photostability of E2-Crimson makes this fluorescent protein useful for multicolor analysis of infectious processes using transgenic cells or tissues expressing other fluorescent proteins such the GFP or orange fluorochromes. Furthermore, E2-Crimson is particularly advantageous in combined exploration of data that rely on the flow cytometry and the two color stimulated emission depletion (STED) microscopy that enables a super-spatial resolution of cellular interactions and other dynamic processes of live cells [24,48].

## Conflict of interest

The authors have declared that no conflict of interests exists between the funders.

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